REMARKS

I. In item number 5 on page 2 of the Office Action, claims 25 and 30-34 were rejected under 35 U.S.C. 103(a) over Johnson et al. (1980).

The rejection is traversed for the following reasons.

In providing justification for the rejection, the Examiner referred to two sentences on page 129 of Johnson et al. (1980) teaching that the titer of astaxanthin in wild-type Phaffia varies from 30-800 μg/g yeast depending on the growth conditions, referring to a 1979 publication by Johnson & Lewis. That passage then also refers to a 1978 publication of Murillo et al. which teaches a Phycomyces strain that produces up to 25 mg/g of β carotene.

The Examiner then concluded that it would have been reasonable for one of ordinary skill in the art to expect that mutations of Phaffia would obtain yields of astaxanthin of more than 800 μg/g yeast.

But that hypothetical argument requires clarification to provide a more accurate comparison of the teachings of the references with that of the instant invention. With a proper comparison, the differences between what is taught in the cited reference and in the instant invention are stark, and will lead to the conclusion there is no suggestion in the cited reference of making and obtaining the claimed invention with a reasonable expectation of success. Also, for example, the teachings of a reference cited in Johnson et al. (1980) cannot be joined with a teaching of Phaffia and astaxanthin.

First, Johnson et al. (1980) does not teach the particular growth conditions necessary to obtain the alleged high levels of astaxanthin. Nevertheless, the best that the Johnson et al. (1980) publication actually teaches for astaxanthin production is 512 μg/g of astaxanthin, see page 129, third full paragraph, first sentence.

As noted on page 126, first paragraph, of Johnson et al. (1980), the astaxanthin content was determined using the method taught in Johnson & Lewis (1979).

Attached hereto is a copy of the Johnson & Lewis (1979) publication. As noted on page 174, eighth full paragraph, astaxanthin amount was obtained using an extinction coefficient of **1600**. That extinction coefficient is important as will be discussed in greater detail hereinbelow.

In Table I on page 176 of Johnson & Lewis (1979), the levels of astaxanthin observed ranged from 212 μg/g through 387 μg/g. In Table III, the astaxanthin yield ranged from 171-652 μg/g. Thus, the highest level of astaxanthin taught in the Johnson & Lewis (1979) publication is 652 μg/g.

In the instant invention, the astaxanthin content was determined using an extinction coefficient of not 1600 but of **2100**. Thus, to make a proper comparison of astaxanthin amounts, a conversion of the calculated astaxanthin amounts is required. That conversion can be done, assuming that all parameters in calculating the astaxanthin content are held constant with the only difference being the change in extinction coefficient by using a conversion formula such as: [(Astaxanthin content in reference x 1600)/2100].

If one takes the highest value of astaxanthin obtained in Johnson & Lewis (1979) of 652 μg/g obtained by using the 1600 coefficient, and converting that amount using a coefficient of 2100, with all other values held constant, that translates to a concentration of 497 μg/g astaxanthin. Thus, using the same coefficient as that of the instant invention, the Johnson & Lewis (1979) publication, as well as the Johnson et al. (1980) publication relied on by the Examiner, teach amounts of astaxanthin less than 500 μg/g. Johnson et al. (1980) per se teach, at best, 390 μg/g astaxanthin, converting 512 μg/g using the 2100 extinction coefficient.

Thus, at best, the Johnson & Lewis (1979) publication teaches that under specific growth conditions, it is possible to obtain no more than 500 μg/g of astaxanthin from a wild-type strain.

On the other hand, the instant claimed invention relates to growth under a very simple set of conditions using the well known YM medium. No particular unusual growth conditions were used as required in the Johnson & Lewis (1979) publication. Thus, it can be concluded that the normal level of astaxanthin in wild-type yeast in Johnson et al. (1980) and Johnson & Lewis (1979), when grown under similar non-optimized conditions as taught in the instant application, should be no more than the 500 μg/g amount using the 2100 extinction coefficient. It also could be concluded that the astaxanthin amounts taught in Johnson et al. (1980) using standard growth conditions should be much less than 500 μg/g.

Thus, the Johnson et al. (1980) reference, in the context of Johnson & Lewis (1979), teaches no more than 500 μg/g of astaxanthin. There is no teaching or suggestion of obtaining higher levels of astaxanthin with a reasonable expectation of success using non-optimized growth conditions, in distinction from the teachings of Johnson & Lewis (1979). Instead, Johnson & Lewis (1979) and thus, Johnson et al. (1980), teach away from using standard growth conditions to obtain enhanced levels of astaxanthin as claimed.

The Examiner then made reference to the Murillo et al. publication.

Attached hereto is a copy of the Murillo et al. publication which teaches strains of Phycomyces that produce carotene, not astaxanthin.

Phycomyces is a yeast that has sexual forms. That fungus thus provides spores which can contain multiple nuclei, see the copy of Eslava et al., PNAS 72 (10):4076-4080, 1975, first full paragraph.

Phycomyces also form mycelia, which means that Phycomyces is a large multinucleate polyploid organism. It is one cell that contains a large number of nuclei, which also means it is a cell that contains a large number of alleles of many genes, Tu & Nalhotra, Microbios. 15(59):15-25, 1976 and Gutierrez-Corona & Cerde-Olnedo, Dev. Genet., 9(6):733-741, 1988.

On the other hand, Phaffia is a unisexual species and does not yield spores. Phaffia does not have a male and female form as Phycomyces does. That means that whereas Phycomyces has the benefit of recombination and independent assortment found in sexual reproduction, Phaffia does not enjoy the benefit of recombination and independent assortment at the level found in Phycomyces.

Also, as noted on page 639 of Murillo et al., right column, second full paragraph, the genetics of carotene biosynthesis in Phycomyces was understood and several genes in the pathway were known. The genetic control of astaxanthin metabolism in Phaffia was not understood to that level.

As noted in the Materials and Methods of Murillo et al., heterokaryons were obtained. As known in the art, heterokaryons are obtained by sexual reproduction of the spores yielding the multinucleate cells that contain nuclei from different cells. Again, as mentioned above, Phaffia does not have a multinucleate form.

As noted on page 640 of Murillo et al., left column, first full paragraph of the Results and Discussion, Murillo et al. teach that sexual interaction of regulatory mutations multiplies stimulatory effects of one gene on another in carotenogenesis. By having multiple nuclei in one cell, the numerous genes and numerous copies of each gene interact to yield greater amounts of carotene. Phaffia does not have a polyploid form.

As noted in Table II on page 640 of Murillo et al., various different crosses were made to yield heterokaryons that produce larger amounts of carotene than found in wild-type strains.

Hence, the crossing of strains to form heterokaryons allows for a greater reassortment and recombination, and because the cells are multinucleate, that unique aspect of Phycomyces enhances the likelihood of complentation from various different genes to yield heterokaryons that can overproduce carotene.

Because Phaffia does not reproduce sexually, there is no opportunity to have the enhanced likelihood of reassortment and recombination of genes as found in Phycomyces. Moreover, because Phaffia does not form multinucleate mycelia, each Phaffia cell contains only a single nucleus and thus there is no opportunity to have large multinucleate cells that contain numerous copies of nuclei and thus numerous copies of individual genes.

Because of the very distinct features of Phaffia and Phycomyces, it is clear that teachings of Murillo et al. cannot be applied to Phaffia. As Phaffia does not have a sexual phase and does not contain a multinucleate cell, there is no reasonable expectation of successfully obtaining mutants of diploid Phaffia that express high levels of astaxanthin.

Attached hereto is the Third Declaration Under 37 CFR 1.132 of Stephen Hiu. In the Third Declaration, Dr. Hiu explains the very distinct biological differences between Phaffia and Phycomyces which makes clear that what operates in Phycomyces will not operate in Phaffia.

The Third Declaration of Dr. Hiu supports the conclusion that the Johnson et al. (1980) publication, along with the two other publications taught therein, Johnson & Lewis (1979) and Murillo et al., neither teach nor suggest the Phaffia mutants overproducing astaxanthin as claimed in the instant application. The disparate nature of the two organisms does not provide any basis to conclude that the teachings of Murillo et al. can apply to Phaffia. Moreover, Johnson & Lewis (1979) and Johnson et al. (1980) provide no guidance to offer any reasonable expectation that the teachings of the references would yield the claimed yeast.

As further support for the instant claims, the Third Declaration of Dr. Hiu also includes additional data relating to other strains of Phaffia with enhanced levels of astaxanthin obtained by practicing the methods taught in the instant application. The strains are in addition to those taught in the First Declaration of Hiu executed 7 November 1997 that teaches strains making more than 2000 μg/g of astaxanthin.

Thus, if one were to presume that wild-type yeast produce about 390 μg/g of astaxanthin taking the figure from Johnson et al. (1980) using the 2100 extinction coefficient, the instant application clearly supports mutant Phaffia which produce more than 700 μg/g and more than 1700 μg/g of astaxanthin, and at least 6 times that of wild-type yeast.

Hence, a prima facie case of obviousness has not been made as to claims 25 and 30-34, and accordingly, the rejection can be removed.

II. In item 6 on page 3 of the Office Action, the Examiner indicated that claims 26-29 would be allowable if rewritten in independent form including all of the limitations of the base claim. Those claims were rejected solely by being dependent on a rejected claim.

Applicants thank the Examiner for acknowledging the patentability of claims 26-29. However, as argued hereinabove, claim 25 clearly is not rendered obvious by Johnson et al. (1980) and thus is patentable.

Hence, all of claims 25-34 are allowable. Accordingly, early indication of allowance is solicited earnestly.

CONCLUSION

Reexamination, reconsideration, withdrawal of the rejection and objection and early notification of allowance are requested respectfully. If any questions remain, the Examiner is requested respectfully to contact the undersigned at the local exchange noted hereinbelow. If any fees are found to be applicable, please charge any additional fees or make any credits to Deposit Account No. 02-1818.

Respectfully submitted,

BELL, BOYD & LLOYD LLC

BY _____

Dean H. Nakamura
Reg. No. 33,981
Customer No. 29180
(202) 955-6851

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Astaxanthin Formation by the Yeast *Phaffia rhodozyma*

By ERIC A. JOHNSON* AND MICHAEL J. LEWIS†

Department of Food Science and Technology, University of California, Davis, California 95616, U.S.A.

(*Received* 5 *January* 1979)

The production of carotenoid pigments by the yeast *Phaffia rhodozyma* depended on the culture conditions. Astaxanthin, the primary carotenoid in this yeast, was produced mainly during the exponential phase of growth. The concentration of carotenes in *P. rhodozyma* remained relatively constant [about 5 μg (g yeast)$^{-1}$] throughout growth in a 1·5% (w/v) glucose medium, but the xanthophyll concentration increased from 90 to 406 μg (g yeast)$^{-1}$ during fermentation. Active xanthophyll synthesis occurred during the period of accelerating growth and after exhaustion of glucose from the growth medium. In media containing more than ... % (w/v) glucose, however, yeast and carotenoid yields were considerably reduced. The p... of the medium affected yeast yields and carotenoid production; the optimum pH was 5·0. At pH 3·5, β-zeacarotene accumulated in *P. rhodozyma*. β-Carotene was the primary carotene in the yeast under all other conditions tested. The optimum temperature for yeast growth and pigment formation was 20 to 22 °C and the best carbon source was D-cellobiose. Oxygen was an important substrate for optimum yields of yeast and astaxanthin; under microaerophilic growth conditions, astaxanthin production was drastically decreased and *P. rhodozyma* accumulated β-carotene and the monoketone echinenone.

INTRODUCTION

Th... d yeasts of the genera *Cryptococcus*, *Rhodotorula*, *Rhodosporidium*, *Sporidiobolus* and *Sporobolomyces* are very similar in their carotenoid composition. They contain β-carotene (β,β-carotene), γ-carotene (β,γ-carotene), torulene (3′,4′-didehydro-β,Ψ-carotene) and torularhodin (3′,4′-didehydro-β,Ψ-carotene-16′-oic acid) as their major pigments (Simpson *et al.*, 1971). Recently, plectaniaxanthin (3′,4′-didehydro-1′,2′-dihydro-β,Ψ-carotene-1′,2′-diol) has been found in *Cryptococcus laurentii* (Bae *et al.*, 1971) and 2-hydroxyplectaniaxanthin in *Rhodotorula aurantiaca* (Liu *et al.*, 1973) which has added some structural diversity to the carotenoids found in this group of fungi. *Phaffia rhodozyma* is a recently discovered yeast (Miller *et al.*, 1976) that is strikingly different from the other pigmented yeasts in producing the carotenoid pigment astaxanthin (3,3′-dihydroxy-β,β-caroten-4,4′-dione) (Andrewes *et al.*, 1976).

Though astaxanthin is rarely found in the fungi [it has occasionally been isolated from the basidiomycetes *Peniophora auranthica* and *Pe. quercina* of the Aphyllophorales (Goodwin, 1972)], it is common in the animal kingdom. It is conspicuously displayed in the plumage of many birds including flamingoes and the scarlet ibis, in marine invertebrates such as lobsters, crabs and shrimps, and in fishes such as trout and salmon, where astaxanthin is responsible for flesh colour. These fish, when raised in pens, often lack desirable red flesh colour. In an earlier study (Johnson *et al.*, 1977) we found that a preparation of *P. rhodozyma* is a potentially important source of astaxanthin for pen-reared salmonids, as the yeast pigment is rapidly accumulated from the feed and deposited in the flesh of rainbow trout.

* Present address: Department of Nutrition & Food Science, Massachusetts Institute of Technology Cambridge, Massachusetts 01239, U.S.A.

† To whom requests for reprints should be sent.

Though some metabolic characteristics of this yeast have been reported (Phaff *et al.*, 1972; Miller *et al.*, 1976), these were not studied in relation to astaxanthin formation. The purpose of this study was to investigate the effect of culture conditions on astaxanthin formation in *P. rhodozyma* with a view to optimizing pigment production.

METHODS

Yeast and culture conditions. The type strain of *Phaffia rhodozyma* (UCD 67–210) was obtained from the yeast culture collection of this department. The yeast was maintained on slants of yeast extract/malt extract agar (YM agar, Difco) at 4 °C.

Flask cultures were grown on an orbital shaker (Environ-Shaker 3597, Lab Line Instruments) at 22 °C in 500 ml baffled side-arm flasks. All shake flask experiments were performed in triplicate. Yeast extract/malt extract broth (YM broth) supplemented with antifoam (FG-10, Dow Corning) at 0·1 ml l^{-1} was the usual growth medium. A medium of yeast nitrogen base (YNB) broth lacking amino acids and ammonium sulphate (Difco) was used in carbon and nitrogen assimilation experiments; carbon and nitrogen sources were added as required. Shake flasks contained 50 ml medium unless otherwise stated. Media were buffered at pH 5·0 with 0·1 M-potassium hydrogen phthalate buffer.

Fermenter cultures were grown in a Virtis 20 l fermenter (model 43-100, Virtis Co., Gardener, N.Y., U.S.A.) at 20 °C using a 14 l working volume, an air flow rate of 8 l min^{-1} and a stirring rate of 400 rev. min^{-1}. The standard medium contained (per litre): Cerelose (CPC International, Englewood Cliffs, N.J., U.S.A.) 20 g; $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0·5 g; $CaCl_2.2H_2O$, 0·1 g; yeast extract (Difco), 2 g; antifoam, 0·1 ml. pH was monitored with a sterilizable pH probe and controlled at pH 4·8 by automatic titration with 4 M-KOH.

Shake flasks were inoculated with 1 % (v/v) and fermenters with 2 % (v/v) of a 20 h washed cell suspension containing about 1·5 mg yeast dry wt ml^{-1}. An additional volume (0·02 %, v/v) of sterile antifoam was added near the middle of the exponential phase of growth to prevent foaming. Yeast growth rate is expressed as the specific growth rate μ (h^{-1}), and in flask culture was estimated during the exponential phase of growth by measuring the increase in absorbance of the culture broth at 600 nm in a Klett photometer (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.). Growth rate in the fermenter was estimated by measuring the increase in cell dry weight as described by Johnson *et al.* (1978). Cultures were harvested by centrifuging after reaching a constant absorbance or dry weight, washed with water, and frozen at −20 °C to await analysis. The yeast yield (Y) is defined as $Y = X_f - X_0$, where X_f and X_0 are the final and initial yeast dry weights (mg ml^{-1}), respectively.

For light induction experiments, *P. rhodozyma* was grown in an orbital shaker incubator equipped with two fluorescent tube lamps which provided 2700 lx at the culture surface. Control flasks were covered with aluminium foil.

Analyses of culture media. Reducing sugar concentration (mg ml^{-1}) in the culture medium was determined with the 3,5-dinitrosalicylic acid reagent (Sumner & Somers, 1949). The rate of dissolution of oxygen into culture media was estimated by the sulphite oxidation method (Cooper *et al.*, 1944). The results are expressed as mmol O_2 dissolved l^{-1} h^{-1}.

Carotenoid extraction and analysis. For routine analyses of astaxanthin, *P. rhodozyma* cell suspensions were mixed with 0·5 mm glass beads, and then vibrated for 3 min in a Braun homogenizer (Bronwill Scientific, Rochester, N.Y., U.S.A.). The broken cells were thoroughly stirred in about 20 vol. acetone, centrifuged, and the pigments in the supernatant were transferred to petroleum ether with the addition of dilute NaCl solution. Astaxanthin concentration in the petroleum ether extract was estimated by measuring the absorbance at λ_{max} (474 nm). The specific absorption coefficient $A^{1\%}_{1cm} = 1600$ (Andrewes *et al.*, 1976) and the formula provided by Davies (1976) allowed the calculation of astaxanthin concentration.

Petroleum ether extracts of carotenoid mixtures to be chromatographed were dried over Na_2SO_4 and concentrated by rotary evaporation at 30 °C in subdued light. The carotenes were separated from the xanthophylls on a MN Kieselgel (Brinkmann Instruments) column by elution with 3 % (v/v) diethyl ether in petroleum ether. Xanthophylls were eluted with acetone. The total concentration of xanthophylls and carotenes in the eluates was estimated by measuring A_{472} for xanthophylls and A_{448} for the carotenes (but A_{422} in cells grown at pH 3·5, see text) using the absorption coefficients $A^{1\%}_{1cm} = 1600$ and 2600, respectively. The individual eluates were sometimes further chromatographed by thin-layer chromatography on aluminium oxide (Alox 25 UV_{254}; Brinkmann Instruments) and silica gel (Silica Gel 60; EM Laboratories) using various combinations of acetone, ethyl ether and petroleum ether as developing solvent mixtures.

Identification of carotenoids. Carotenoids which had been purified to chromatographic homogeneity were characterized by their electronic absorption spectrum, by co-chromatography with identical or related pure carotenoids in two solvent systems and by their mass spectrum (if sufficient material was available).

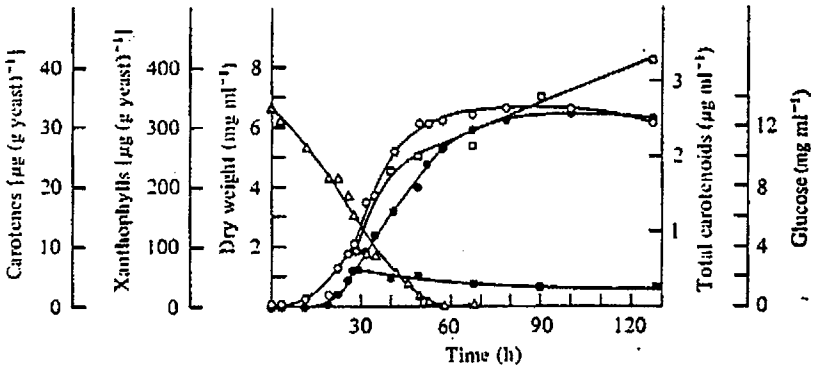

Fig. 1. Production of carotenoids by *P. rhodozyma* in fermenter batch culture. Yeast growth (○), total carotenoid formation (●), xanthophyll (astaxanthin) formation (□), carotene synthesis (■) and glucose utilization (△). The growth medium initially contained 1·5% (w/v) D glucose.

Visible absorption spectra were recorded in acetone, and concentrations of carotenoids were calculated using the specific absorption coefficients provided by Davies (1976). The mass spectra were determined on a Finnegan 3200 instrument at 220 °C with an ionization voltage of 70 eV.

Chemicals. All chemicals were, where possible, of analytical grade. D-Mannitol, L-arabinose and D-xylose were obtained from ICN Pharmaceuticals, Cleveland, Ohio, U.S.A.; cellobiose was from Sigma, glucono-δ-lactone from Merck; maltose from Calbiochem; sucrose, succinic acid, D-glucose, potassium hydrogen phthalate, phosphate salts and all solvents from Mallinckrodt, St. Louis, Mo., U.S.A.; and 3,5-dinitrosalicylic acid from Eastman Kodak Co.

RESULTS

Growth and astaxanthin production

In fermenter batch culture, growth of *P. rhodozyma* began after a 10 h lag and a constant dry weight of yeast was reached after about 80 h (Fig. 1). Termination of growth coincided with the exhaustion of glucose from the medium. Astaxanthin was found to be the major xanthophyll in all samples of yeast taken during the fermentation and was produced mainly during the exponential growth period. Its production slowed soon after cessation of growth. The concentration of xanthophylls in the cells increased from 92 to 225 μg g^{-1} during the period of exponential growth (30 to 40 h) and then increased only slightly to about 260 μg g^{-1} in the next 20 to 30 h. On exhaustion of glucose, the concentration of xanthophylls increased steadily until 128 h when a final concentration of 406 μg g^{-1} was obtained. In contrast, the concentration of carotenes in *P. rhodozyma* during the lag and exponential phase was fairly constant at 6 μg g^{-1} and decreased to 3 μg g^{-1} in the stationary phase. The primary carotene identified in all samples during growth was β-carotene.

Effect of pH on growth and pigment formation

In a preliminary screening of buffers, it was found that 0·1 M-potassium hydrogen phthalate and 0·1 M-sodium phosphate buffered well over the necessary range of pH values and allowed good growth and pigmentation of *P. rhodozyma*. Citrate buffer was unsatisfactory because it inhibited growth. Lactate buffer was also unsuitable because a rise in pH of the culture broth occurred late in the exponential phase of growth, probably due to the utilization of lactate by *P. rhodozyma* (Miller *et al.*, 1976).

In phthalate or phosphate buffer, the final yield of yeast was only slightly affected by pH in the range 3·8 to 7·5 (Fig. 2) in shake flasks. The growth rate of *P. rhodozyma* was much more affected by pH and was highest at pH 5·8. The yield of astaxanthin was also affected by medium pH; a maximum yield of 2·0 μg ml^{-1} was obtained at pH 5·0 and at this pH the concentration of astaxanthin in *P. rhodozyma* was also highest [510 μg (g yeast)$^{-1}$]. Gener-

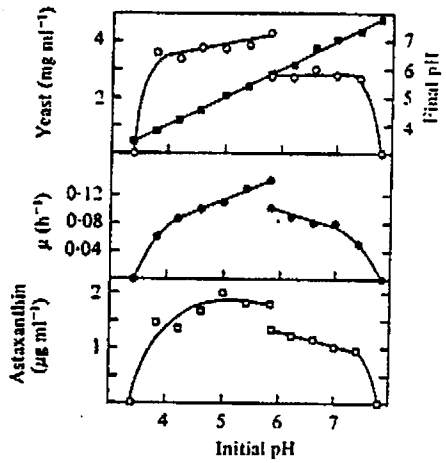

Fig. 2. Effect of pH on carotenoid formation and growth of *P. rhodozyma* in shake flasks buffered with 0·1 M-potassium hydrogen phthalate or 0·1 M-sodium phosphate (pH 5·8 to 7·8). Final yeast yield (○), yeast growth rate (●), astaxanthin yield (□) and final pH (■).

Table. 1. *Effect of pH on growth and astaxanthin formation of P. rhodozyma in fermenter culture*

pH of medium	Yeast yield (mg ml⁻¹)	Growth rate, μ (h⁻¹)	Astaxanthin [μg (g yeast)⁻¹]	Total xanthophylls [μg (g yeast)⁻¹]	Total carotenes [μg (g yeast)⁻¹]
6·5	6·03	0·14	325	332	11·0
5·5	9·68	0·12	336	339	15·0
4·5	11·79	0·16	387	385	6·5
3·5	5·96	0·09	212	219	15·0

ally within the pH range 4·0 to 7·0 the choice of buffer (potassium hydrogen phthalate or sodium phosphate) had a greater effect on the parameters tested than did pH. Sodium phosphate buffer was slightly inhibitory and the yield of yeast, yeast growth rate and astaxanthin formation were all lower in phosphate buffer than in phthalate buffer at the same pH (5·8).

The influence of pH on carotenoid production was also studied in fermenters since the pH could be controlled by automatic titration so that the effects of buffers were eliminated. Of the four pH values studied (Table 1), the optimum was found to be pH 4·5 where the maximum yield of cells, the highest growth rate and the maximum production of astaxanthin were obtained. Analysis of the pigments showed that astaxanthin was the dominant xanthophyll present at each pH value. However, the absorption spectrum of the carotene fraction from yeast grown at pH 3·5 was strikingly different from that obtained at other pH values (Fig. 3). The primary carotene present at pH 3·5 was β-zeacarotene whereas β-carotene dominated in yeast grown at the other pH values (see Table 2). After purification, these pigments were conclusively identified by their absorption spectra, chromatographic characteristics and mass spectra. β-Zeacarotene could not be found in cells grown at pH 4·5.

Influence of temperature on growth and pigmentation of P. rhodozyma cultured in shake flasks

The final yield of yeast was relatively constant at growth temperatures of 22 °C and below, but the yield decreased considerably at temperatures above 22 °C, the optimum for growth rate (Fig. 4). The highest temperature at which growth was observed was 27·5 °C; at this temperature *P. rhodozyma* increased its mass about twofold after inoculation and

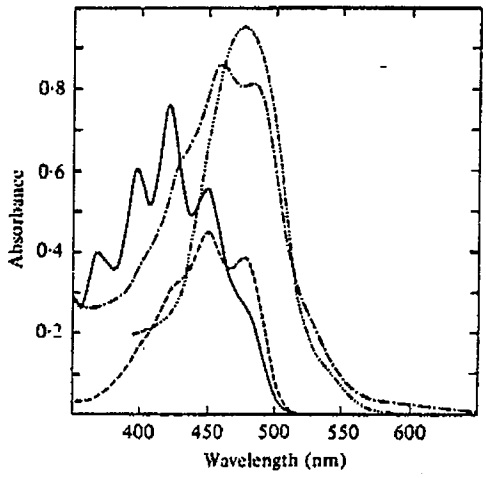

Fig. 3. Visible absorption spectra (in acetone) of the carotenes isolated from *P. rhodozyma* grown at pH 3·5 (———) and pH 4·5 (— — —) and of the total carotenoid extract of cells grown under aerobic (· · · — · · · —) or microaerophilic (— · — ·) conditions.

Table 2. *Analysis of carotenes isolated from P. rhodozyma grown in a fermenter at pH 3·5 or 4·5*

		pH 4·5		pH 3·5	
Carotene	Structure	μg isolated	% of total*	μg isolated	% of total*
β-Carotene	β,β-Carotene	27	77·0	trace	~1
β-Zeacarotene	7′,8′-Dihydro-β,ψ-carotene	0	0	40	90
γ-Carotene	β,γ-Carotene	2·7	7·7	1·5	3·0
Neurosporene	7,8-Dihydro-γ,γ-carotene	1·7	5·0	2·5	5·0
Lycopene	γ,γ-Carotene	3·5	10·0	trace	~1

Total yield of carotenes: pH 3·5, 15 μg (g yeast)$^{-1}$; pH 4·5, 7 μg (g yeast)$^{-1}$. The concentrations of ...nophylls were 219 and 385 μg (g yeast)$^{-1}$, respectively.

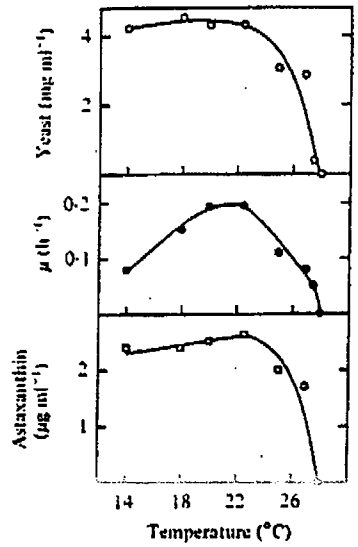

Fig. 4. Effect of temperature on growth and pigmentation of *P. rhodozyma* grown in shake flasks. Symbols as in Fig. 2.

Table 3. *Effect of carbon source on growth and pigmentation of P. rhodozyma in shake flask culture*

The growth medium was 50 ml 0·1 M-phthalate-buffered yeast nitrogen base medium (see Methods), containing 0·6% (w/v) Bacto-peptone and 200 mg carbon (supplied as the various sugars). The values represent the mean of two determinations. Carbon sources were sterilized separately from the basal medium. No growth occurred in the basal medium without the addition of a carbon source.

Carbon source	Growth rate, μ (h^{-1})	Yeast yield (mg ml^{-1})	Yeast yield [mg (mg carbon)$^{-1}$]*	Astaxanthin yield (μg ml^{-1})	Astaxanthin yield [μg (g yeast)$^{-1}$]
D-Maltose	0·14	3·63	0·91	1·86	512
D-Cellobiose	0·10	3·48	0·87	2·27	652
Sucrose	0·19	3·72	0·93	1·89	508
Succinate	0·09	2·66	0·67	1·33	500
D-Mannitol	0·16	3·68	0·92	1·80	489
D-Xylose	0·04	1·21	0·30	0·58	479
L-Arabinose	0·05	3·30	0·83	1·25	379
Glucono-δ-lactone	0·10	1·48	0·37	0·80	541
D-Glucose	0·20	3·85	0·96	1·62	421
D-Glucose†	0·21	6·46	0·81	1·11	171

* Assuming all carbon utilized. † 800 mg carbon [4% (w/v) glucose].

then stopped growing. The astaxanthin concentration in yeast grown at all the temperatures tested was constant (about 480 μg g^{-1}). Insufficient yeast was obtained at 27·5 °C to estimate the concentration of astaxanthin, but the cells were very pale. All the acetone extracts gave visible absorption spectra typical of astaxanthin. The concentrations of carotenes in the yeast also remained constant at about 7 μg g^{-1}.

Growth and pigmentation of P. rhodozyma grown on various carbon sources

Cellobiose supported more pigmentation of *P. rhodozyma* [652 μg (g yeast)$^{-1}$] than any of the other carbon sources tested (Table 3). The other disaccharides, maltose and sucrose, also promoted high pigmentation. Sucrose and glucose promoted more rapid growth of *P. rhodozyma* (μ 0·19 h^{-1}) than the other carbon sources. Although succinate and glucono-δ-lactone supported slow growth and rather sparse yields of yeast, these compounds promoted high concentrations of astaxanthin in *P. rhodozyma*. The sugar alcohol D-mannitol supported good yeast growth and pigmentation. The pentoses L-arabinose and D-xylose were utilized but resulted in slow growth and carotenoid production. D-Glucose supported a high rate of growth but only a moderate yield of astaxanthin, especially when present at a higher concentration (4%, w/v).

Influence of glucose concentration and shaking on growth and pigmentation of P. rhodozyma

As *P. rhodozyma* is a fermentative yeast, growth and pigment production were studied in a wide range of glucose concentrations in YM medium in shake flasks. The final yield of yeast per g glucose utilized decreased significantly with increasing glucose concentrations in shake flasks. The yield of astaxanthin per g yeast followed a remarkably similar pattern (Fig. 5). However, because the total yield of yeast increased substantially in the high glucose media, more astaxanthin (μg ml^{-1}) was produced. At concentrations of glucose above 10 mg ml^{-1}, the efficiency of biomass and astaxanthin production decreased steadily. Above about 40 mg glucose ml^{-1}, the yeast yield per g glucose and astaxanthin yield per g yeast were less affected by increasing glucose concentrations than below 40 mg ml^{-1} (Fig. 5).

The production of carotenoids was also studied in a fermenter in medium containing 5% glucose (Fig. 6). Glucose was not completely utilized after 126 h in this medium; yeast yields were reduced to about half those obtained in the 1·5% glucose fermentation and the concentration of astaxanthin in *P. rhodozyma* decreased to 350 μg (g yeast)$^{-1}$ (compare

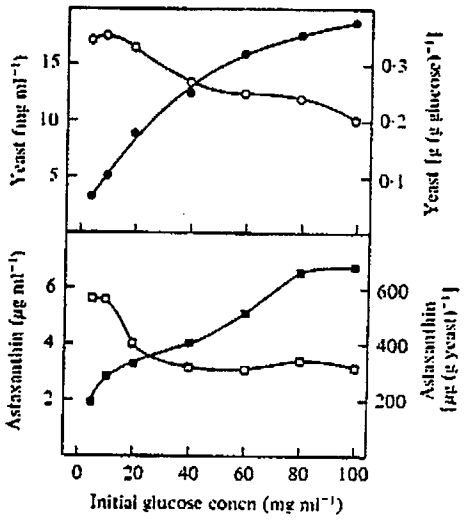

Fig. 5. Effect of glucose concentration on growth and pigmentation of *P. rhodozyma*. Final yeast yield (●), yeast yield per g glucose (○), astaxanthin yield (■) and astaxanthin yield per g yeast (□). Calculations corrected for residual glucose. The growth medium contained (per litre in 0·1 M-phthalate buffer, pH 5·0): 3 g yeast extract, 3 g malt extract, 6 g peptone (YM basal broth) and various concentrations of D-glucose.

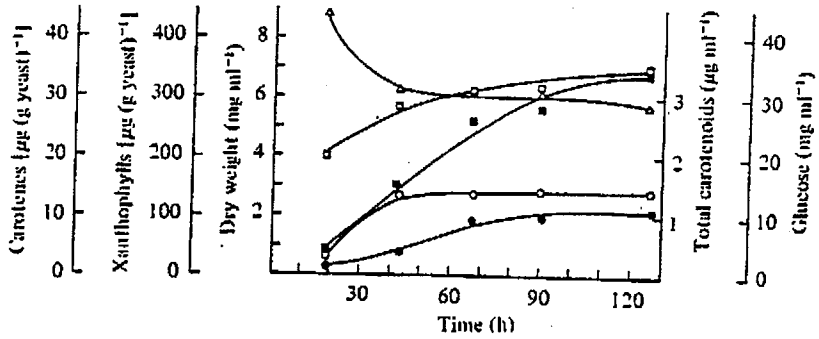

Fig. 6. Effect of 5% glucose on carotenoid formation and growth of *P. rhodozyma* in fermenter batch culture. Yeast growth (○), total carotenoid formation (●), xanthophyll (astaxanthin) formation (□), carotene synthesis (■) and glucose utilization (△).

Fig. 1). However, the concentrations of total carotenoid pigment in the cells grown at the two glucose concentrations were almost the same (about 400 μg g⁻¹); this was because the concentration of carotenes was 12-fold higher in the cells grown in the high-glucose medium. The primary carotene present in yeast grown in the 5% glucose medium, from all fermenter samples, was β-carotene. In addition, approximately 2·5% of the total carotenes in the cells was found to be β-zeacarotene. The absorption spectrum of the xanthophyll fraction, in all samples, was identical to that of astaxanthin and therefore we did not analyse this group of pigments for concentrations of individual carotenoids.

Effects of relative aeration on growth and pigmentation

To study the effects of aeration on growth and carotenoid production in *P. rhodozyma*, we varied the volume of medium in the shake flasks between 25 and 200 ml and their shaking rates from 50 to 200 rev. min⁻¹ to produce a range of aeration rates. The dissolution rates of oxygen into a sulphite-containing solution indicated that a wide range of aeration rates was achieved (from 3·6 to 108 mmol O_2 dissolved l^{-1} h^{-1}).

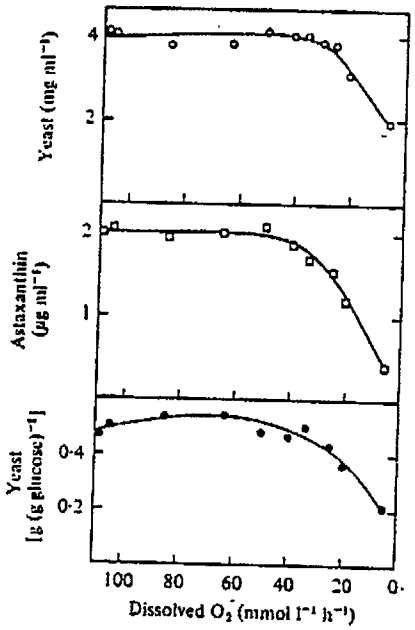

Fig. 7. Effect of aeration on carotenoid formation and growth of *P. rhodozyma*. Final yield (○), yield per g glucose (●) and astaxanthin production (□). The abscissa represents the air supply as mmol O_2 supplied l^{-1} h^{-1}, obtained in 0·5 l baffled shake flasks by using different medium volumes (25, 50, 100 and 200 ml) and shaking speeds (50, 100, 150, 200 rev. min^{-1}).

Table 4. *Carotenoids of P. rhodozyma grown under microaerophilic or aerobic conditions*

Carotenoid	Structure	Individual carotenoids isolated from cells (% of total carotenoid present)*	
		Microaerophilic	Aerobic
Astaxanthin	3,3′-Dihydroxy-β,β-carotene-4,4′-dione	26	87
Phoenicoxanthin	3-Hydroxy-β,β-carotene-4,4′-dione	4	6
3-Hydroxy-3′,4′-didehydro-β,ψ-caroten-7-one	—	9	<1
3-Hydroxyechinenone	3-Hydroxy-β,β-carotene-4-one	<1	2
Echinenone	β,β-Carotene-4-one	27	3
β-Carotene	β,β-Carotene	33	2
Other carotenes	—	<1	<1

* Total carotenoid yield: aerobic, 509 μg (g yeast)$^{-1}$; microaerophilic, 244 μg (g yeast)$^{-1}$.

The yields of cell mass and astaxanthin were fairly independent of oxygen dissolution rates except at the lowest aeration values, i.e. less than 30 mmol O_2 l^{-1} h^{-1} (Fig. 7). Below this level, the yields of yeast and astaxanthin were significantly reduced; at the lowest oxygen dissolution rate (3·6 mmol l^{-1} h^{-1}) the yield of yeast decreased from the usual value of about 4·0 mg ml^{-1} to 2·0 mg ml^{-1} and the yield of astaxanthin decreased from approximately 2·0 to 0·3 μg ml^{-1}.

Because low aeration drastically influenced the concentration of carotenoids in *P. rhodozyma*, we incubated *P. rhodozyma* without an air supply in the fermenter. Under these conditions, the yeast tripled its biomass but then grew no more. Addition of ergosterol and Tween 80 to the medium did not promote growth. The harvested yeast was low in total

carotenoids (244 $\mu g\ g^{-1}$) and the visible absorption spectrum of the total carotenoid extract was not typical of astaxanthin (see Fig. 3). The concentration of xanthophylls was 163 $\mu g\ g^{-1}$ and of carotenes 81 $\mu g\ g^{-1}$. The concentrations of individual carotenoids are given in Table 4. Under these microaerophilic conditions, comparatively little astaxanthin was produced; it made up only 26% of the carotenoid mixture compared with nearly 90% under aerobic growth conditions. The primary carotenoid synthesized under anaerobic conditions was β-carotene. Echinenone was also produced in much higher amounts anaerobically than in aerobically grown yeast.

Because low aeration and high glucose in the growth medium caused significant reductions in the efficiency of astaxanthin production, we decided to combine these effects. In a medium containing 4% (w/v) glucose and with O_2 supplied at 5·0 mmol $l^{-1}\ h^{-1}$, the specific growth rate of *P. rhodozyma* was 0·1 h^{-1} and the yield of yeast was only 0·05 mg (g glucose)$^{-1}$. The cells were tan rather than pink and contained only 30 μg total carotenoid g^{-1}. On analysis the total pigment extract showed a visible absorption spectrum similar to β-zeacarotene. Chromatography showed that the yeast contained little astaxanthin but proportionately higher concentrations of less polar pigments (probably carotenes) including β-zeacarotene.

Light and carotenoid production

Phaffia rhodozyma was grown in triplicate shake flasks in YM medium in the dark or with hi light intensity (2700 lx). The yield of yeast in the dark- and light-grown cultures was 3·[illegible] and 3·5 mg ml^{-1}, respectively. Observation of the shake flasks after growth suggested that the light-grown cultures synthesized more astaxanthin, because the cells had a redder hue. This may have been due to different relative concentrations of the carotenoids present, since the assay of astaxanthin gave only a slightly increased mean, 538 $\mu g\ g^{-1}$ compared to 510 $\mu g\ g^{-1}$ for the dark-grown culture.

Effect of nitrogen source and complex media on pigmentation in P. rhodozyma

The concentration of ammonium sulphate in the range 0·25 to5 mg ml^{-1} had little effect on the yeast growth rate, final yeast biomass or on carotenoid production in YNB medium su[illegible]lemented with 1% D-glucose. The substitution of $(NH_4)_2HPO_4$ or peptone for $(NH_4)_2$-[illegible] at various concentrations also did not affect these values. However, when increasing concentrations of yeast extract (0·1 to 10 mg ml^{-1}) were added to a vitamin-free medium (vitamin-free yeast base, Difco), there was an increase in pigmentation from 156 to 524 $\mu g\ g^{-1}$. Similarly, when *P. rhodozyma* was grown in fermenter culture on complex media – (i) brewer's malt wort diluted to a specific gravity of 1·020 or (ii) addition of 1·0 l of colourless tomato pressings to 2·0 l of the standard medium – the carotenoid yields were 712 and 814 $\mu g\ g^{-1}$, respectively, after 60 h growth.

DISCUSSION

Astaxanthin formation in *P. rhodozyma* is clearly growth-associated, although its production does not exactly coincide with increase in biomass. The growth-associated production of astaxanthin contrasts with results found with *Sporobolomyces roseus* (Bobkova, 1965) and *Rhodotorula glutinis* (Vecher & Kulikova, 1968), where carotenoid production occurred only after yeast growth had stopped; it is also common in the Phycomycetes (e.g. *Phycomyces blakesleeanus*) for the primary period of carotenoid synthesis to follow cessation of growth.

Phaffia rhodozyma is the only carotenogenic yeast that ferments glucose (Miller *et al.*, 1976). It would be expected that growth at low dissolved oxygen concentrations and/or high glucose levels would promote fermentative metabolism and possibly affect carotenoid production. When the supply of oxygen to *P. rhodozyma* was reduced to low levels there

zyma. Final yeast yield
scissa represents the air
using different medium
. min^{-1}).

c or aerobic conditions

Individual carotenoids isolated from cells (% of total carotenoid present)*	
Microaero-philic	Aerobic
26	87
4	6
9	< 1
< 1	2
27	3
33	2
< 1	< 1

44 μg (g yeast)$^{-1}$.

of oxygen dissolution
$l^{-1}\ h^{-1}$ (Fig. 7). Below
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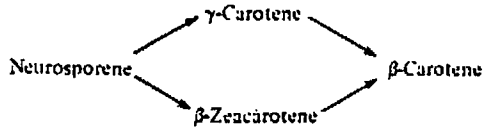
tion of ergosterol and
east was low in total

was a decrease in the yield of yeast per g glucose utilized, which suggested that *P. rhodozyma* was fermenting. This was accompanied by a decrease in astaxanthin concentration and an accumulation of β-carotene. Similarly, when *P. rhodozyma* was cultured with increasing concentrations of glucose, fermentative growth was indicated by decreased yields of yeast per g carbon utilized and this was accompanied by decreases in astaxanthin concentration. These results show that astaxanthin production is inhibited under fermentative conditions. This is supported by the fact that glucose, which is readily fermented by *P. rhodozyma*, promoted relatively low levels of astaxanthin production. Cellobiose, however, which can only be used aerobically by *P. rhodozyma* (Phaff *et al.*, 1972), stimulated relatively high astaxanthin production as did succinate, which may be directly utilized aerobically by the tricarboxylic acid cycle.

Changes in the lipid content of aerobically and anaerobically grown *Saccharomyces cerevisiae* have been interpreted as a reflection of the state of mitochondrial development (Jakovicic *et al.*, 1971). The inability to form ergosterol in mutants of *S. cerevisiae* (Bard *et al.*, 1974) is due to lesions in the biosynthesis of porphyrins rather than direct lesions in the synthesis of sterols, which implies the requirement of an active respiratory chain for the synthesis of ergosterol. It is possible that an active respiratory chain is also needed in the biosynthesis of carotenoids, since these share many enzymic steps with sterols in their formation from acetyl-CoA. Exposure of anaerobically grown cells to oxygen causes a rapid induction of the sterol-synthesizing enzyme, 3-hydroxy-3-methyl-glutaryl CoA reductase (Berndt *et al.*, 1973). Further, since carotenoids have been reported to be located primarily in the mitochondria in certain fungi (Heim, 1946), this location may also indicate their site of synthesis.

Very little is known about the formation of xanthophylls in micro-organisms. It is generally assumed that hydroxyl functions at C-3 and C-3′ of the carotenoid skeleton arise from the incorporation of molecular oxygen by carotene hydrocarbons. The evidence for this hypothesis is indirect (see Britton, 1976). Nothing is known about the introduction of oxygen functions at C-4 and C-4′ except that it is likely that oxo groups are formed through the hydroxy intermediates (Britton, 1976). When *P. rhodozyma* was cultured with minimal oxygen supply, the astaxanthin yields were greatly reduced, and the yeast tended to accumulate β-carotene as well as the monoketone echinenone. These results suggest that the hydroxyl functions in astaxanthin are formed only in the presence of oxygen and that carotenes and echinenone are formed under conditions of low aeration. When we aerated anaerobically grown stationary phase cells, there was no detectable change in their astaxanthin content.

β-Zeacarotene accumulated in cells of *P. rhodozyma* grown under adverse environmental conditions. This pigment was not originally detected in the analysis of the pigments of *P. rhodozyma* by Andrewes *et al.* (1976). Its isolation suggests that the well known alternative route for β-carotene synthesis is operative in *P. rhodozyma* thus:



β-Zeacarotene accumulates in cells of Rhodotorula (Simpson *et al.*, 1964) and *Phycomyces blakesleeanus* in the presence of inhibitors. Because β-zeacarotene usually accumulates in cells only under adverse conditions, e.g. in the presence of inhibitors such as diphenylamine or in stressful environments, it may be regarded as an indicator of inefficient carotenoid biosynthesis. Its formation can perhaps be rationalized by a hypothesis of Goodwin and his coworkers, (McDermott *et al.*, 1974) who postulate that the synthesis of zeaxanthin by *Flavobacterium* spp. involves an enzyme complex with two active sites, each of which acts on a carotenoid 'half molecule' in synchrony and with equal efficiency, i.e.

desaturation or cyclization of each half molecule proceeds at the same rate. Under abnormal conditions, however, the individual sites may not act in synchrony and asymmetrical products such as β-zeacarotene may result. Further, genetic evidence has shown that there is an enzyme complex with two cyclases for the formation of β-carotene in Phycomyces (De la Guardia *et al.*, 1971).

In contrast to many other micro-organisms, light does not stimulate carotenogenesis in *P. rhodozyma*. The biosynthesis of astaxanthin was, however, greatly enhanced by growth in the presence of tomato wastes. The high yield of carotenoid obtained with the tomato supplement suggests that carotenoid precursors may enter the cell and enhance carotenoid production. These results indicate the possibility of commercial production of astaxanthin by *P. rhodozyma* using citrus or vegetable wastes as adjuncts in the fermentation.

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Carotene-Superproducing Strains of *Phycomyces*

F. J. MURILLO, I. L. CALDERÓN, I. LÓPEZ-DÍAZ, AND E. CERDÁ-OLMEDO
Departamento de Genética, Facultad de Ciencias, Universidad de Sevilla, Seville, Spain

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Production of β-carotene by wild-type *Phycomyces blakesleeanus* can be stimulated by light, chemicals, regulatory mutations, and sexual interaction between mycelia of opposite sex. Through genetic manipulations, we have isolated strains which have simultaneously and constitutively incorporated several of these stimulatory effects. In the dark and in a simple medium, some of the strains produce up to 25 mg of β-carotene per g (dry weight), or about 500 times the wild-type production under the same conditions. High lycopene-producing strains have also been isolated by using *carR* mutants, which are blocked in the conversion of lycopene to β-carotene. These strains should be useful in both industrial production of these pigments and basic research related to carotenogenesis.

The production of β-carotene by *Phycomyces blakesleeanus* depends on media and culture conditions but is generally low. In the dark, this fungus produces on the order of 50 μg of β-carotene per g (dry weight), making it inappropriate for industrial use (16).

β-Carotene production can be stimulated in several ways. Photoinduction results in an accumulation of about 500 μg/g under blue-light intensities of 2 W/m^2 (3), but light stimulation presents considerable practical difficulties in large-scale application.

Several chemicals stimulate carotenogenesis when added to the medium (22); up to 2,000 μg/g is accumulated in the presence of vitamin A (14), but the required concentrations of the vitamin are prohibitively large; up to 4,000 μg/g has been observed under the best conditions in the presence of β-ionone (16).

Mutations in the gene *carS* result in β-carotene contents of up to 4,000 μg/g in the dark. The *carS* mutants are still sensitive to vitamin A, but a double mutant, strain S106, has been obtained which reaches 6,000 μg/g. The new mutation, *car-102*, makes S106 insensitive to vitamin A. The stimulatory channel activated by vitamin A has thus become constitutive in this strain (18).

In the *Mucorales*, the interaction between mycelia of opposite sex leads to increased carotenogenesis through formation of trisporic acids (2, 6; A. Prieto, C. Spalla, M. Bianchi, and G. Briffi, Commun. Int. Ferment. Symp. London, p. 38, 1964). Mixed cultures of *Blakeslea trispora* strains of opposite sex in the presence of β-ionone have been considered promising for β-carotene production. However, it is difficult to maintain appropriate sex ratios in large cultures (10).

In *Phycomyces*, sexual stimulation occurs in single mycelia, called intersexual heterokaryons, which contain a mixture of nuclei of opposite sex (5). Such heterokaryons produce more than 400 μg of β-carotene per g (18), have a peculiar morphology, with formation of small aerial hyphae or pseudophores, and are unstable; they tend to segregate the components in homokaryotic form.

The genetics of carotene biosynthesis in *Phycomyces* has been reviewed (9; E. Cerdá-Olmedo and S. Torres-Martínez, Pure Appl. Chem., in press). Mutants in gene *carB* form phytoene, and mutants in gene *carR* produce lycopene (17, 21). All the intermediates from phytoene to β-carotene may be obtained in *Phycomyces*, either through the use of certain genetic combinations (1, 12) or through the addition of inhibitors (13, 19).

The objective of this work was to obtain *Phycomyces* strains that would yield high contents of β-carotene or other carotenes when grown on simple media in the dark.

MATERIALS AND METHODS

The strains of *P. blakesleeanus* used in this work are listed in Table 1. References to details about isolation, genotypes, and carotene production are given in the same table.

Heterokaryons were produced by using a previously described method (20). In the text, the two heterokaryon components are separated by an asterisk.

Cultures of homokaryons and heterokaryons were initiated as reported earlier (18). For all quantitative studies, cultures were grown on solid minimal medium

TABLE 1. *Strains of P. blakesleeanus used in this work*

Strain	Genotype	Main carotenoid produced (μg/g, dry wt)	Reference
NRRL1554	Wild type (+)	β-Carotene (56)	18
M1	*carS43* (+)	β-Carotene (4,160)	18
S106	*carS42 car-102 mad-103* (−)	β-Carotene (5,595)	18
H7	*carR51* (+)	Lycopene (1,200)	This work
S136	*carR127* (+)	Lycopene (650)	This work
C9	*carR21* (−)	Lycopene (2,470)	21 and this work

(15), with glucose as carbon source, at 22°C for 4 days. In a few cases, cultures were grown on potato-dextrose agar (7), since its low cost would be an advantage in large-scale application. To observe distinct colonies, the minimal medium was supplemented with 1 mg of yeast extract per ml and acidified to pH 3.3.

The extraction of carotenes and their chromatographic separation and identification have been described previously (11, 12, 21).

Mutants were isolated after treatment with 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) per ml in pH 7.0 citrate-phosphate buffer, as previously described (8).

RESULTS AND DISCUSSION

Intersexual heterokaryons with *carS* mutations. Sexual interaction and regulatory mutations multiply each other's stimulatory effect on carotenogenesis when acting simultaneously in the intersexual heterokaryon M1 * S106. Some of the heterokaryotic mycelia contain 500 times more β-carotene than does the original wild type (Table 2).

These heterokaryotic mycelia are highly variable in β-carotene content, presumably reflecting variations in nuclear ratios. They are much less stable than the heterokaryons described by Heisenberg and Cerdá-Olmedo (15), since they tend to separate into mycelial patches of different colors and produce unexpectedly high proportions of homokaryotic spores. Sexual type is only one of the many differences in genetic background among the constituent nuclei, which are descended from very different wild types. The reasons for the instability are, thus, unclear. In any case, instability is a major drawback for the practical application of a strain.

Intersexual heterokaryons with balanced lethal mutations. The disadvantages of the intersexual heterokaryon M1 * S106 would presumably disappear in the diploid M1/S106. We have made considerable, but fruitless, efforts to isolate diploids from different *Phycomyces* heterokaryons.

An alternative would be to introduce recessive lethal mutations in both components of the heterokaryon. To this effect, spores from M1 * S106 mycelia were treated with NTG to a survival level of about 0.1%. Most nuclei are thus inactivated, and most survivors are homokaryotic. Among the heterokaryotic survivors, there are many that cannot segregate either or both components in homokaryotic form, owing to the introduction of recessive lethal mutations (8). A total of 117 survivors of the treatment, taken from among the most brightly colored, were tested for segregation by streaking their spores on acid medium. The heterokaryons S218 * S219 and S242 * S243 were obtained in this way. They produced considerable quantities of β-carotene (Table 2), and no homokaryons were found among their progeny, although they still showed a wide range of nuclear ratios, leading to variations in β-carotene content.

Variation in nuclear ratios could be limited if the lethal mutations were not totally recessive. If both heterokaryon components had mutations or sets of mutations that made mycelia with more than 70% of the corresponding nuclei inviable, nuclear proportions would be limited to the 30 to 70% range. Heterokaryotic S218 * S219 spores were treated with NTG, and a search for stable strains led to the isolation of S244 * S245 and S246 * S247. These heterokaryons are apparently very uniform and have high β-carotene contents (Table 2).

TABLE 2. *Production of β-carotene in intersexual heterokaryons M1 * S106 and their derivatives*

Strain	β-carotene (μg/g, dry wt)[a]
M1 * S106, mycelium A	14,340
M1 * S106, mycelium B	18,450
M1 * S106, mycelium C	20,325
M1 * S106, mycelium D	20,500
M1 * S106, mycelium E	25,342
S218 * S219	16,000 (12,620)
S242 * S243	15,600 (9,000)
S244 * S245	19,120 (14,120)
S246 * S247	13,470 (25,230)

[a] Numbers in parentheses correspond to potato-dextrose agar cultures. The others correspond to the usual minimal medium cultures.

High lycopene production. The lycopene content of strains C9 and H7 (Table 3) largely exceeds the β-carotene produced by the wild type. There is an apparent feedback regulation (18), so that the lack of the end product, β-carotene, stimulates the pathway.

The heterokaryon H7 * C9 produces no pseudophores and does not surpass the lycopene content of strain C9 alone (Table 3). This total lack of sexual stimulation supports the concepts that both pseudophore formation and carotenogenesis are activated by trisporic acids and that these acids derive from β-carotene (24). To exhibit sexual stimulation, a heterokaryon must thus be able to synthesize at least some β-carotene.

Strain S136 was isolated after treatment of spores of strain NRRL1554 with NTG. This strain contains lycopene (Table 3) as the main carotene, but also γ-carotene (110 μg/g), β-carotene (45 μg/g), and smaller amounts of phytofluene, ζ-carotene, and neurosporene. It presumably carries a mutation in gene *carR*, resulting in decreased cyclase activity, responsible for the production of the cyclated carotenes γ-carotene and β-carotene (12).

The heterokaryon S136 * C9 produces pseudophores, exhibits very intense red colors, and has high lycopene content. Spores of S136 *C9 were treated with NTG, and a search for stable heterokaryons resulted in the isolation of S183 * S184, S185 * S186, and S187 * S188 (Table 3). Although stability was achieved, optimum production was not. Further mutagenesis should bring this about.

Final comments. The strains obtained in this work reach very high carotene levels when grown on simple media in the absence of light or exogenous chemical stimulation. They should be particularly useful in the development of industrial processes for carotene production. There is room for further improvements and extensions. New stimulatory mutations, similar to mutation *car-102* in strain S106, could be introduced in *carS* (+), *carR* (+), and *carR* (−) genetic backgrounds and serve as the basis for a repetition of the process described in this paper. The intersexual heterokaryons could also carry mutant and wild-type alleles of genes *carB* and *carR* so that all the intermediates from phytoene to γ-carotene could be produced (1, 12). Media and culture conditions optimal for carotenogenesis in other organisms (10) should be tried out, since we have made no effort in this direction.

The strains obtained in this work should be particularly useful in the development of in vitro systems for carotene biosynthesis and for the isolation of mRNA's and proteins involved in the process (4, 23).

TABLE 3. *Formation of lycopene in homokaryons and intersexual heterokaryons containing carR nuclei*

Strain	Lycopene (μg/g, dry wt)
C9	2,470
H7	1,200
S136	650
H7 * C9 mycelium A	1,950
H7 * C9 mycelium B	2,575
H7 * C9 mycelium C	2,675
S136 * C9 mycelium A	9,780
S136 * C9 mycelium B	10,550
S136 * C9 mycelium C	12,540
S136 * C9 mycelium D	12,570
S136 * C9 mycelium E	14,600
S183 * S184	6,946
S185 * S186	5,830
S187 * S188	7,101

The successive increases in carotene content described in this paper support the hypothesis of the independence of the stimulatory effects of *carS* mutations, vitamin A, and sexual interaction (18).

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Genetics

Meiosis in *Phycomyces*

(linkage/map units/centromere)

ARTURO P. ESLAVA*, MARIA ISABEL ALVAREZ*, AND MAX DELBRÜCK

Division of Biology, California Institute of Technology, Pasadena, Calif. 91125; and *Max Planck Institut für Molekulare Genetik, Ihnestrasse 63-73, 1 Berlin 33, West Germany

Contributed by M. Delbrück, July 28, 1975

ABSTRACT **A four-factor cross between two strains of *Phycomyces* involving two auxotrophic, one color, and the mating type marker is described. Samples of 40 germspores from 84 individual fertile germsporangia were characterized. The results show: (*i*) The germspores of a germsporangium are derived from one meiosis in approximately 78% of the cases. (*ii*) The four markers are on separate chromosomes. They are nonselective. (*iii*) Analysis of a large sample of germspores from 106 pooled germsporangia confirms that the four markers are unlinked. (*iv*) From the ditype/tetratype ratios it is inferred that each marker is located about 15 map units from its centromere.**

Phycomyces, like other mucoraceous fungi, is heterothallic (1). The two mating types, (+) and (−), are indistinguishable morphologically; when they grow near each other a series of mutually induced biochemical and morphological changes takes place (2–5) culminating in the formation of a highly multinucleate zygospore (6). The zygospore, after a long dormancy (2–6 months, depending on the strains), produces a germsporangium containing germspores containing one to six haploid nuclei each, like those in the vegetative sporangium.

The great majority of the germspores are homokaryotic, possibly because they are formed from protospores containing only one nucleus which undergoes mitotic divisions to bring the number of nuclei up to the known multinucleate state (7).

A feature affecting all previous work on sexual genetics was the lack of regularity of the genotypes in the progeny; often the germspores from a single germsporangium were all infertile, usually several of the expected genotypes were missing, and the ones found varied greatly in number. These irregularities made the analysis of the sexual crosses a difficult task. Burgeff in 1928, based on very limited data from crosses involving morphological markers, found that recombinants are formed and suggested that although many hundreds of nuclei of both mating types enter into the zygospore, in general only one diploid nucleus undergoes meiosis followed by a number of postmeiotic mitoses yielding, in the germsporangium, 7,000 to 15,000 germspores. The rest of the nuclei entering into the zygospore were presumed to abort, either after fusing to form diploids or in the initial haploid state. The cytological studies to establish the karyological events have been inconclusive and the genetic data have been too limited to clarify the sexual genetics of *Phycomyces* and, in general, of the Mucorales. Both aspects have been reviewed recently (6, 8).

With the use of new auxotrophic and color markers and parental strains yielding a shorter dormancy of the zygospores, conditions for high and reproducible germination of the zygospores have been established (9). Clear evidence was found that apogamic nuclei do not contribute to the progeny. In addition, the data suggested that a standard meiotic process is operating in the generation of recombinants (9, 10).

To investigate more precisely the nature of the recombinational process in *Phycomyces*, a four-factor cross involving two auxotrophic, a color, and the mating type markers has been analyzed.

MATERIALS AND METHODS

Strains. The parental strains used in the four-factor cross and their pedigree are displayed in Fig. 1.

Media. SIV was used as a minimal medium. It is similar to SI (4) except that SIV contains 2 g/liter of asparagine-H_2O as a source of nitrogen instead of monosodium glutamate. Various complete media and supplements were used as required (9).

Culture Methods and Analysis. The procedure for the sexual cross and the analysis of the genotypes of the progeny has been described (9).

EXPERIMENTAL

The cross: germination data

Fig. 1 shows the pedigree of the parental strains used in the four-factor cross here reported. The two wild types, UBC21 (+) and NRRL1555 (−) from which the parentals were derived, presumably differ in genetic background, since they were isolated in different places at different times. The (+) parental strain of our cross (C242) resulted from a backcross designed to make the genetic background more isogenic.

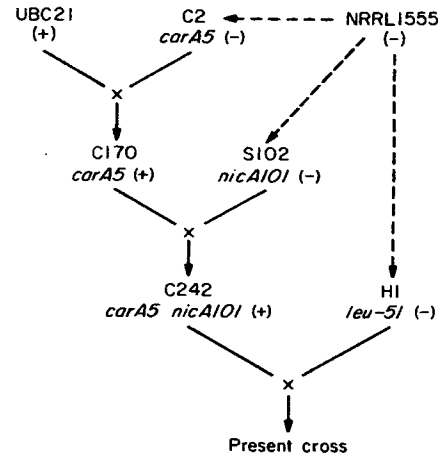


FIG. 1. Pedigree of the strains used. UCB21 and NRRL1555 are the wild types. The genotype is shown below each strain. Continuous lines indicate sexual crosses. Broken lines indicate nitrosoguanidine mutagenesis. *car* designates genes involved in β-carotene synthesis.

Table 1. Progeny samples from 11 germsporangia

Class of germsporangium[b]	Minimal no. of meioses[c]	Viable spores (average)	Genotypes[a]: I sex II *leu* III *nic* IV *car*																Mt[e] het
			(+ + − −)[d]	(− − + +)[d]	(− + + −)	(+ − − +)	(+ + + −)	(− − − +)	(− + − −)	(+ − + +)	(+ − + −)	(− + − +)	(− − + −)	(+ + − +)	(+ − − −)	(− + + +)	(− − − −)	(+ + + +)	
1T	1	3000									17								20
1T(6)	1	8000				40													
$2T_{nr}$[f](7)	1	1300		8		31										17	22		
$2T_{r}$[g](10)	1	8000																	
3T(14)	1	6000	8						19	3									
4T(28)	1	7000	9		10			11		10									
3T(2)	2	4000			19							17					4		
4T(7)	2	2700	10			3		20					7						
5T(5)	2	2500		14				1			8	14					2		1
6T(3)	2	3500			2	2			6		9							2	18 1
7T(1)	3	3000		1			2	16	8		4						1		8

[a] The 16 possible genotypes are arranged in pairs of reciprocals. The wild-type allele is represented as +, the mutant allele as −. In the sex marker + represents mating type (+) and − mating type (−).

[b] These class designations indicate the number of genotypes found in the sample taken. In parentheses the number of germsporangia in this class. The first germsporangium listed is unusual in yielding about 50% mating type heterokaryons. One germsporangium (not listed) yielded 100% mating type heterokaryons.

[c] A single meiosis produces at most two genotypes with the same allele of any one gene. By this criterion some samples require at least two, and one requires at least three meioses.

[d] Parental genotypes.

[e] Mating type heterokaryons.

[f] $2T_{nonreciprocal}$.

[g] $2T_{reciprocal}$.

A total of 128 zygospores were set out on water agar. One hundred twelve (88%) germinated and produced germsporangia. Among the germinated zygospores, 84 (75%) yielded germsporangia with viable spores. The remainder yielded no viable spores. Microscopic inspection showed that these sterile sporangia did contain a large number of spores. An additional set of 109 germsporangia were pooled, and the germspore count gave an average of 15,000 per germsporangium. The viability of these pooled germspores on rich acid medium (9) was found to be 40%.

The shortest dormancy, defined as the time elapsed from the day at which mating plates were inoculated to the germination of the first zygospore, was 100 days, about 40 days longer than the shortest dormancy in the cross between the two progenitor wild types, UBC21 and NRRL1555. The reason for this lengthening of the dormancy is probably that C242 was selected, in the backcross of its origin, for a particular combination of markers and not for shortest dormancy. It has been shown that dormancy is determined polygenically (9).

Number of meioses per germsporangium

The germsporangia are classified according to the number of different genotypes found in the samples taken (1T, 2T, ...). In the case of a germsporangium yielding two genotypes, the two genotypes may be either a reciprocal pair (class $2T_{reciprocal}$) or they may not be reciprocal (class $2T_{nonreciprocal}$). This classification scheme ignores the occurrence of mating type heterokaryons. Table 1 gives, as an example, the analysis of 11 germsporangia according to this classification. For each germsporangium the number of segregants in each genotypic class is given.

In a single meiosis the two alleles of each gene segregate 2:2 (except for conversions). Therefore, each allele of any gene is represented in not more than two of the meiotic products. Germsporangia are classified as resulting from one meiosis if they are compatible with this rule. Otherwise two or more meioses must be assumed. Table 1 shows (first column) the numbers of germsporangia in each class. The majority of them, 65 of 83, are compatible with a single meiosis; 17 require at least two meioses, and only one at least three. These numbers are lower limits, since more than the indicated number of meioses may have occurred in any one germsporangium, with none of the products of the extra meioses being found in the sample taken. However, this source of error is certainly small in view of the fact that the largest class found is that exhibiting four genotypes compatible with a single meiosis. On a random basis this would be a very unlikely event in a four-factor cross with 16 possible genotypes (one out of 35 cases).

Linkage tests

Linkage Between Markers. The average aspects of the recombination mechanisms in *Phycomyces* can be studied either by analyzing a large sample of germspores from a pool of germsporangia or by analyzing samples of germspores from individual germsporangia. The first procedure is simpler but it gives information only about the linkage of the markers. The second procedure determines the frequencies of the different types of germsporangia (1T, 2T, ..., etc.), giving, therefore, more information about the meiotic process. Only the presence or absence of the various genotypes in the samples tested are counted in order to minimize the effects of secondary mechanisms, such as asynchrony in the postmeiotic divisions of the four haploid products (9).

Table 2 shows the distribution of the parental alleles in the progeny using the two procedures. Both procedures show

Note: Performing your original search, ***phycomyces number of nuclei***, in PubMed will retrieve 3 citations.

1: Microbios. 1976;15(59):15-25.

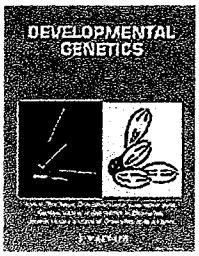
Links

The formation of sporangiospores in Phycomyces.

Tu JC, **Malhotra SK**.

The multinucleate state of the vegetative spores of Phycomyces blakesleeanus arises as a consequence of cleavage of cytoplasm containing a variable number (1-6) of pre-existing nuclei. No nuclear division or incorporation of 3H-thymidine was detected during maturation of the spores.

PMID: 979660 [PubMed - indexed for MEDLINE]



Developmental Genetics

What is RSS?

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Article

Genetic determination of sporangiophore development in *Phycomyces*

Félix Gutiérrez-Corona, Enrique Cerdé-Olmedo *

Departamento de Genética, Facultad de Biologia, Universidad de Sevilla, Sevilla, Spain

*Correspondence to Enrique Cerdé-Olmedo, Departamento de Genética, Facultad de Biologia, Universidad de Sevilla, Apartado 1095, E-41080 Sevilla, Spain

KEYWORDS

Phycomyces blakesleeanus • developmental mutants • phorogenesis • sexual reproduction • light • carotene

ABSTRACT

The mycelium of the fungus *Phycomyces.* essentially a giant multinucleate cell, produces two kinds of asexual reproductive structures, called macrophores and microphores, and a succession of structures for sexual reproduction. Following the treatment of spores with N-methyl-N′-nitro-N-nitrosoguanidine, conditional *imb* mutants have been isolated that form no macrophores at 26°C, but do at 14°C. At the restrictive temperature, few *imb* mutants (2 of 13) develop microphores, and none is able to complete the sexual cycle. This suggests that genes responsible for macrophorogenesis are involved in microphorogenesis and in sexual development as well. Light reduces macrophorogenesis and totally abolishes microphorogenesis in the wild type under the conditions of our experiments. These photomorphogenetic effects require the normal function of genes *madA* and *madB*, which are responsible for phototropism. Light inhibits microphorogenesis in the two *imb* mutants that form microphores at the restrictive temperature. Genetic alterations of carotenogenesis lead to an excess of microphores and a scarcity of macrophores in the dark, but they have little influence on vegetative reproduction in the light.

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